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NITROGENASE IX

EFFECT OF THE MgATP GENERATOR ON THE CATALYTIC AND EPR PROPERTIES OF THE ENZYME IN VITRO

LAWRENCE C. DAVIS and WILLIAM H. ORME-JOHNSON *

Department of Biochemistry and the Center for the Study of N_2 Fixation, College of Agricultural and Life Sciences, and the Institute for Enzyme Research, University of Wisconsin-Madison, Madison, Wisc. 53706 (U.S.A.)

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Summary

Nitrogenase (nitrogen:(acceptor) oxidoreduction, EC 1.7.99.2) of *Clostridium pasteuranium* is very sensitive to the ratio of MgADP/MgATP in dithionite oxidation assays. Variation of concentration of creatine kinase, an ATP-regenerating enzyme, can be used to control the ratio of ADP/ATP and thereby the dithionite oxidation activity of nitrogenase. The in vitro properties of nitrogenase support the suggestion of Haaker (Haaker, H., deKok, A. and Veeger, C. (1974) Biochim. Biophys. Acta 357, 344—357) that in vivo the nucleotide ratio and not the electron supply normally regulates nitrogenase activity.

In EPR experiments it has been shown that the "steady state" varies as a function of the concentration of creatine kinase. The spectral differences are interpreted as being a function of the ratio of MgADP/MgATP obtained in the pseudo steady-state condition, which occurs as a result of variation in relative rates of ATP-utilizing and ATP-generating reactions, that is, the relative nitrogenase and creatine kinase activities. Implications of these finding for interpretation of previously reported kinetic and EPR studies are discussed.

Introduction

It was suggested, on the basis of in vitro experiments [1], that levels of MgATP and MgADP are important for regulation of nitrogenase (nitrogen:(acceptor) oxidoreductase, EC 1.7.99.2) activity in vivo. Recently evidence has been presented that ADP/ATP ratio ** are indeed controlling for in vivo nitro-

* To whom correspondence should be addressed.

** Unless otherwise indicated, in this paper ADP/ATP ratio means [MgADP]/[MgATP].

genase activity [2] if the earlier in vitro experiments are correct in their estimate of the sensitivity of nitrogenase to ADP/ATP ratio. We have sought to reexamine the role of ADP/ATP ratio in regulation of nitrogenase activity, using purified enzyme and a coupled assay system to provide steady-state assays of activity.

According to the studies of Tso and Burris [3] the binding of MgADP to the Fe protein (component II) of nitrogenase is somewhat stronger than the binding of MgATP, with binding constants of 5 and 10—20 μM, respectively. Ljones [4] has shown that MgADP inhibits dithionite oxidation by nitrogenase in an approximately competitive manner with a K_i for MgADP near the K_m of MgATP. For these studies he used substrate levels of ATP and followed progress curves of inhibition. Routine assays of reduction of nitrogenase substrates generally make use of an ATP-generating system since ADP is potent inhibitor of the nitrogenase system [5] and several mol of ATP are consumed for each mol of substrate reduced. Control of the ratio ADP/ATP is also necessary for proper interpretation of the appearance of new EPR signals during the course of substrate utilization [6,7,14].

The most commonly used ATP-regenerating system consists of creatine-*P*, creatine kinase and set amounts of ATP and Mg^{2+}. The absolute levels of each of the assay constituents used vary from one laboratory to another [8—13]. It is generally assumed that the regenerating system responds in an ideal fashion, so that the steady-state concentration of the essential substrate remains constant at the initial level while the concentration of possible inhibitors (e.g. ADP and MgADP) is negligible over the course of the assay. We will show that this assumption, although adequate for routine assays, is not a correct description of the actual assay. This becomes important in studies of nitrogenase activity at very low levels of ATP [8,12] or during EPR experiments [6,7,14] where 10—100 μmol ATP are consumed per min per ml reaction, at 30° C.

One thus needs a regenerating system capable of very high activities with few side reactions, non-inhibitory products, thermodynamics favoring linearity over a significant fraction of substrate utilization, and reasonable cost. No known system meets all these criteria fully, but with an awareness of its limitations, the creatine kinase system is the best available. The rule of creatine kinase in nitrogenase assays, and the influence of ADP/ATP ratios of nitrogenase activity are the subjects of this communication.

Materials and Methods

Disodium ATP was obtained from Sigma or PL Biochemicals, and ADP from Sigma. Concentrations of ATP determined enzymatically with hexokinase, glucose, glucose-6-phosphate dehydrogenase and NADP [20] agreed well with those specified by the manufacturers. ATP labeled with ^{14}C was purchased from Amersham Searle and was stored frozen in ethanol/water. Its specific activity was 52 Ci/mol. Dithionite was obtained from British Drug Houses. It was prepared for assays as a stock solution of 100 mM dithionite in 50 or 100 mM Tris · HCl or Tris/morpholinopropane sulfonic acid buffer of pH greater than 8. It was found to be stable for at least a week if kept in serum stoppered vials.

Creatine kinase was obtained from Sigma Chemical Co.; creatine phosphate

was from Pierce Chemicals. Other enzymes were obtained from Sigma or Boehringer. Morpholinopropane sulfonic acid (MOPS) was from Sigma; Tris was a special enzyme grade from General Biochemicals. All other chemicals were of reagent grade, obtained from commercial sources. DEAE paper was a Whatman product purchased from Curtin-Matheson.

Nitrogenase was prepared by a modification of the purification procedure of Tso et al. [15]. Neither the Mo-Fe protein (component I) nor the Fe protein (component II) oxidized dithionite at a detectable rate in the absence of the other. Specific activities in the dithionite oxidation assay at pH 7.4 were approx. 1 μmol/min per mg and approx. 0.5 μmol/min per mg for the two components, respectively. When dithionite oxidation assays or acetylene reduction assays are carried out at pH 6.8 rather than pH 7.4, the specific activities of both components are increased about 2-fold. Both were checked for EPR-detectable contaminants prior to use in further experiments, and appeared to be essentially pure by this criterion. Gel electrophoresis indicated that they were of purity with respect to proteins comparable to that reported by Tso et al. [15] although of lower specific activity.

Creatine kinase was specified by Sigma as having an activity of 35 μmol/min per mg ADP phosphorylated at pH 7.4 in Sigma's assay system. True specific activities were determined by simultaneous variation of creatine-*P* and ADP in constant ratio [16]. For assays of nitrogenase activity, potassium morpholinopropane sulfonate was used since Cl^- or other commonly used anions are inhibitors of creatine kinase [17].

Dithionite oxidation assays of nitrogenase activity were carried out by the method of Ljones and Burris [18] but following the oxidation of dithionite at 350 nm using an experimentally determined ϵ_M of 1300 so that a wider range of concentrations could be used than would be the case if the reaction were followed at 315 nm. Assays were carried out in 0.5 ml buffer in 1 ml nominal volume cuvettes sealed with rubber serum stoppers. These were flushed and evacuated four times under N_2 purified over hot copper catalyst [7]. Dithionite was added to the reaction mixture with a 10 μl Hamilton syringe. The rate of spontaneous dithionite hydrolysis under these conditions was less than 0.1%/min for at least an hour. The apparent rate in the absence of added ATP was usually even less after addition of the nitrogenase components and was disregarded in calculations of enzymatic activities.

Assays for determination of the specific activities of nitrogenase components were done using the final concentrations of 50 mM potassium morpholinopropane sulfonate adjusted to a final pH 7.4, 5 mM magnesium acetate, 2.5 mM ATP, 1 mM dithionite, 30 mM creatine-*P* and 0.2 mg/ml creatine kinase. Assays for determination of distribution of ^{14}C between ATP and ADP were similar except that approx. 10^6 cpm/ml of 50 Ci/mol [^{14}C]ATP was added prior to the flushing with N_2. For these assays, aliquots of the reaction mixture were withdrawn during the course of the dithionite oxidation while the cuvette remained in the spectrophotometer. Samples were immediately spotted on DEAE paper, quenched with ethanol and chromatographed as described by Morrison and Cleland [16], using ammonium formate (pH 3 with 5 mM EDTA) for development.

When nitrogenase was to be added to assays in a constant ratio of compo-

nents it was prepared by mixing aliquots of the two components in heavy-wall 7 × 150 mm pyrex tubes capped with serum stoppers. Such tubes could be rapidly frozen and thawed to avoid cold inactivation of the component II. As obtained from the purification procedure (in 50 mM Tris · HCl, pH 8, 1 mM dithionite) the reconstituted enzyme was stable for several working days or when subjected to several cycles of rapid freezing and thawing.

For EPR experiments, the EPR tubes containing the assay mixture were capped with serum stoppers and flushed and evacuated as for cuvettes. Dithionite was added to give a final concentration of approx. 10 mM in a final volume of 300 μl. The enzyme was then added with a gas-tight Hamilton syringe. Samples were frozen after appropriate times by plunging the tubes in cold isopentane (−140°C) as previously described [7].

Results

Alternate MgATP-generating systems

One of our initial concerns was to find an ATP-regenerating system which would permit us maximal flexibility in conditions of assay. We therefore compared creatine kinase with pyruvate kinase and acetate kinase, in terms of kinetic parameters, efficiency and cost. We found that pyruvate reacts with dithionite so that piruvate kinase is unacceptable [4]. The kinetic constants of acetate kinase [19], and the cost of pure enzyme made this a poor choice. We were thus left with creatine kinase and sought to exploit its properties as best we could.

The kinetic parameters of creatine kinase are well defined [20,21]. ATP synthesis is strongly favored and so long as the kinase is in reasonable excess over the nitrogenase equilibrium the ADP concentration will remain low until the creatine phosphate is nearly all used up. Typical enzyme assays are carried out with a low level of nitrogenase and an arbitrary concentration of creatine kinase (about 0.2 mg/ml). As discussed below this may lead to misinterpretation of results when higher levels of nitrogenase are used without a corresponding increase in creatine kinase levels. In addition, the capacity of creatine kinase to phosphorylate ADP decreases rapidly at high pH as the apparent K_m rises. We used the assay method of Schimerlik and Cleland [20] to compare enzyme activity at pH 7 and pH 8 in our buffer. With 400 μm ADP the apparent K_m for creatine-*P* is 0.5 mM at pH 7 and 4 mM at pH 8. With 20 mM creatine-*P* the apparent K_m for ADP remains constant at 25 μM. This means in practical terms that the creatine kinase activity in a nitrogenase assay will be dependent on pH, creatine-*P* concentration and the level of ADP deemed acceptable for the study at hand. The enzyme is also inhibited by a wide range of anions and forms deadend complexes with creatine and ATP [17,20]. For precise work, one must thus determine the activity of the creatine kinase under conditions to be used for nitrogenase activity and insure that it is indeed in excess.

Properties of the creatine kinase : nitrogenase system

In Fig. 1 we show the effect of varying the level of creatine kinase, holding nitrogenase constant under conditions (pH 7.4, excess nitrogenase component II) such that there is a nearly optimal ATP : $2e^-$ ratio (5.5 for ATP utilization

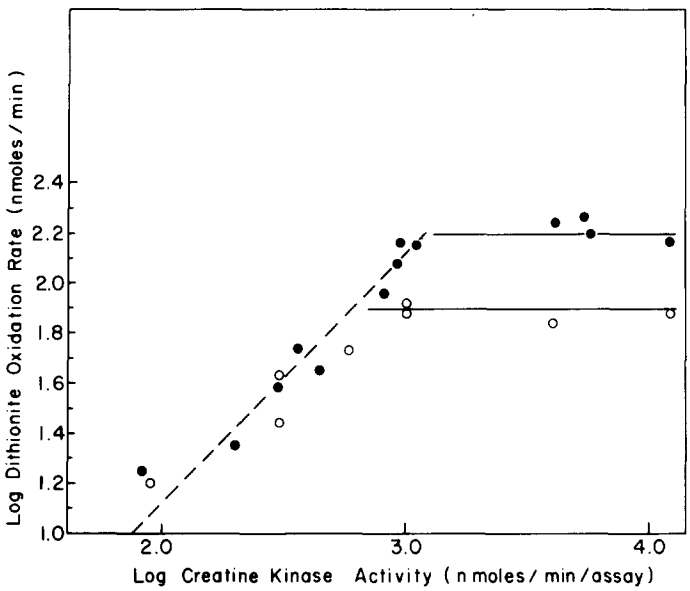

Fig. 1. Dithionite oxidation as a function of creatine kinase concentration. Assays were carried out as described in Materials and Methods. Concentration of creatine kinase is expressed as units of activity present, based on extrapolation of activity to V by the method of Morrison and Cleland [16]. The specific activity of this preparation of creatine kinase was found to be 70 nmol/min per μg and the highest concentration used in the assay was 0.2 mg in a final volume of 0.525 ml. All dilutions of kinase were made in 1 mg/ml bovine serum albumin and the concentration of kinase plus albumin was always above 0.2 mg/ml in the final assay. A plot of log activity vs. log of kinase concentration is used for convenience in presenting the data over a wide range of creatine kinase concentrations. •, rates of dithionite oxidation obtained using 0.3 mg component I and 1.6 mg component II; ○, rates obtained using one-half the amount of each component. Solid lines are drawn to indicate the expected 2-fold difference of nitrogenase acitivity with excess creatine kinase; the broken line is drawn with an intercept of log 5.5, which is the ratio of ATP hydrolyzed to dithionite oxidized using substrate levels of [^{14}C]ATP (see Fig. 3).

by nitrogenase) [11]. The amount of nitrogenase used is typical of assays for acetylene reduction carried out in the upper linear range for this assay. Apparent linearity was obtained for about 20 min at the highest level of creatine kinase. Typical acetylene reduction assays with 2—10-fold less nitrogenase activity present are nearly linear for more than 30 min [22] although they are usually only followed for 15 min [13].

Reducing the amount of kinase per assay by 10-fold from the standard level of 0.2 mg/ml had no significant effect on initial and little effect on steady-state velocity under these conditions. When the kinase was lowered a further 4-fold, inhibition became apparent and the steady-state rate of dithionite oxidation decreased considerably.

Measurement of the ratio of ADP to ATP in the assay system as a function of time, which measures the steady-state capacity of kinase to cope with the ATP hydrolysis by nitrogenase, showed a marked change with occurred in parallel with the change of dithionite oxidation rate (Fig. 2). Each pair of data points in Fig. 2 represents the steady-state (not initial) rate of dithionite oxidation and the corresponding ADP level produced by varying the creatine kinase concentration.

An alternative method of presenting the data is to set the most rapid rate of

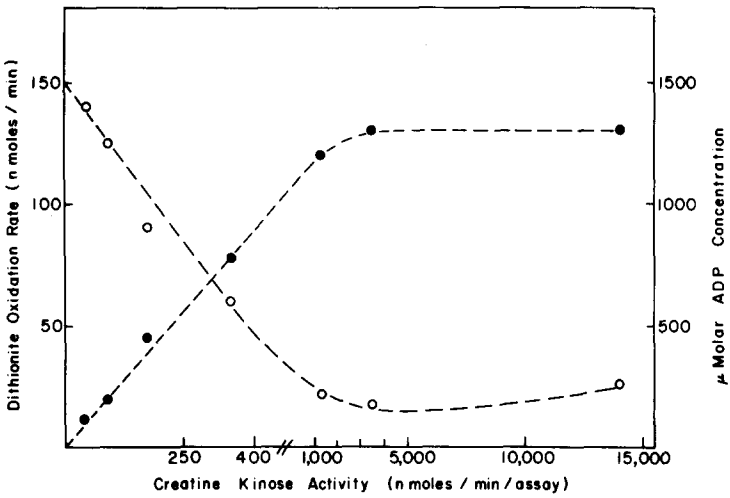

Fig. 2. Variation of dithionite oxidation as a function of ADP concentration. Assays were carried out as indicated in Materials and Methods with the same preparation of creatine kinase as used for Fig. 1. ●———●, dithionite oxidation rate; ○———○, ADP concentration. Note change of scale on abscissa between 400 and 1000 units.

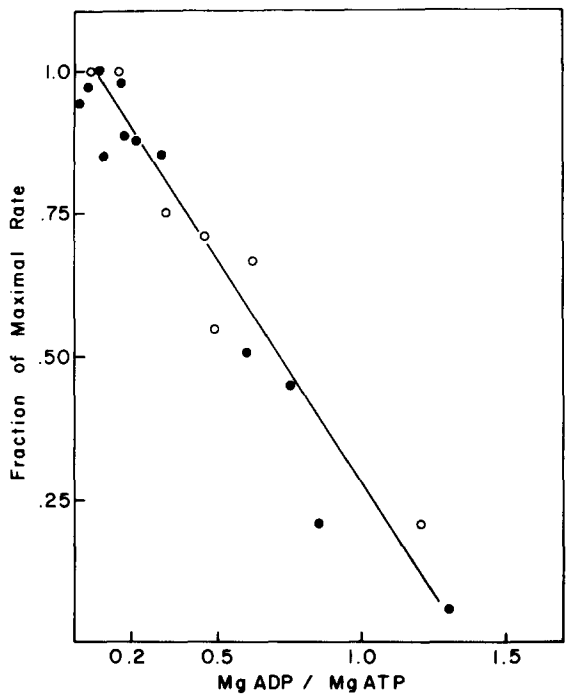

Fig. 3. Dithionite oxidation as a function were done as described in Materials and Methods and Fig. 2 except that two different levels of component I were used. On the graph: ●, excess component II; ○, limiting component II. The solid line indicates the relationship of ADP/ATP ratio to dithionite oxidation activity as found in Fig. 2.

dithionite oxidation equal to 100% and compare relative activities as a function of the ADP-to-ATP ratio. Fig. 3 shows such a plot for time course of single assays determined with two different ratios of nitrogenase components. Relative inhbition was very similar in both cases even though the ATP : $2e^-$ ratio varied from 5.5 to > 10 and the dithionite oxidation rate increased about 2-fold yielding a net 4-fold increase in rate of ATP utilization when component I was increased.

For assay with a relative excess of component II, the enzyme mix was the same as Fig. 2. For assay with an excess of component I, an additional 2.25 mg of component I was used. The concentrations of Mg^{2+}-nucleotides were calculated from their stability constants. The total amount of dithionite oxidized for exhaustion of the ATP-regenerating system was found to be twice as much for the limiting I case as for excess I, and the time to 90% exhaustion of the energy source was 20 min for limiting I, compared to 5 min with excess I present.

Discrimination between ADP and MgADP as inhibitors of nitrogenase

We have carried out assays using substrate level ATP with varying levels of magnesium to determine whether MgADP or uncomplexed ADP [23] is an inhibitor of nitrogenase activity. When levels of magnesium were limiting in the assay, the inhibition by accumulation of ADP was much less than when there was excess magnesium present. However, the inhibition was greater than would be expected if MgADP were the sole inhibitor. ATP was 8.0 mM with 2.4 mM magnesium for the first series (Fig. 4A) and 7.4 mM ATP with 8.1 mM Magnesium for the second series (4B). ADP levels are indicated on the figure with progress curves offset to indicate the difference in curvature between conditions, by making the assumption that 5.5 mol of ATP are hydrolyzed per mol of dithionite oxidized (see Fig. 1) and setting accordingly the initial absorbancies of those samples to which ADP was added. The offset in time is arbitrary. Broken lines in part B indicate the expected slope for the addition of the specific amount of ADP (as derived from initial velocity of assay with that amount of added ADP) not correcting for net depletion of ATP in the control assay. This shows that the ratio ADP/ATP, not the total ADP concentration, is the critical variable.

We calculated from the patterns of inhibition obtained when ADP was present primarily as the free or at the complexed form, Fig. 4A vs. Fig. 4B, that MgADP is approximately five times more potent than free ADP as an inhibitor of the dithionite oxidation reaction of nitrogenase. In part A there is seen a good agreement between the entire course of a reaction to which no ADP was added and the initial velocities of reaction to which varying amounts of ADP were added. In contrast to this behavior with low levels of Mg^{2+}, when excess Mg^{2+} was present as in part B, the control reaction became rapidly inhibited. In those assays of part B to which ADP was added there was both an effect on initial rate and a progressively more rapid onset of inhibition as ADP concentration was increased.

Measurement of [^{14}C]ADP/ATP ratio vs. dithionite oxidation rates under the conditions of Fig. 4 confirmed our estimate of the potency of ADP vs MgADP as an inhibitor of nitrogenase. When 9.1 mM ATP plus 2.2 mM Mg^{2+} was used, the inhibition observed was 3-fold less than if there had been sufficient Mg^{2+}

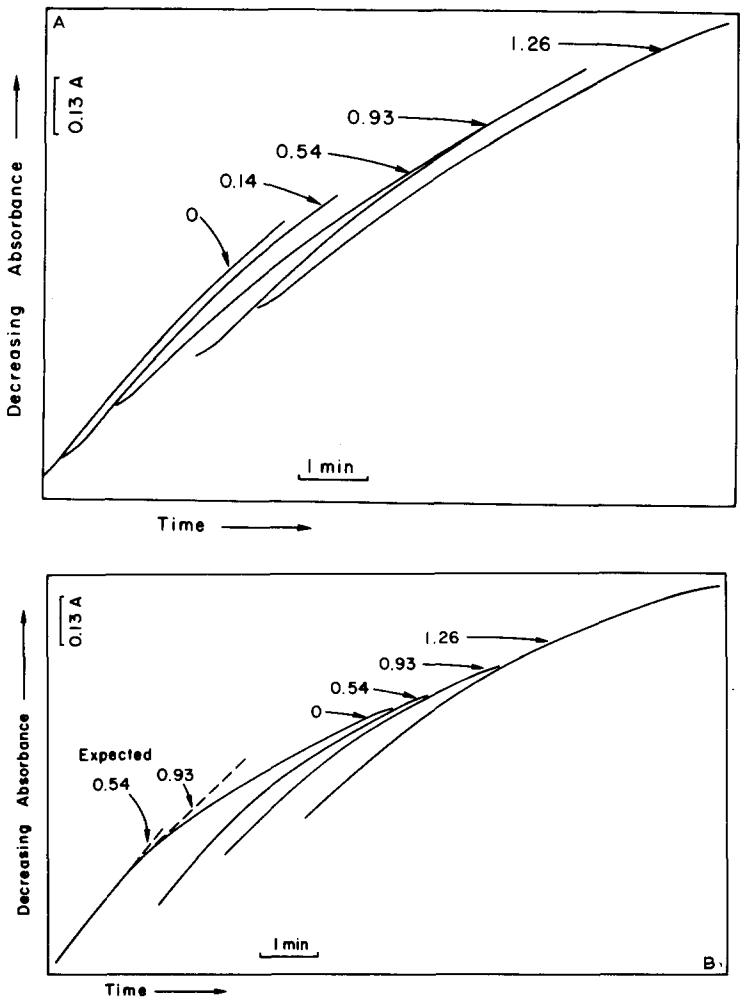

Fig. 4. Rates of dithionite oxidation as a function of the concentration of ATP, ADP and magnesium. Assay conditions and enzyme as in Fig. 1 but omitting creatine-*P* and creatine kinase. Nitrogenase was a mixture of 0.15 mg component I and 0.8 mg component II per assay. Part A, 2.4 mM Mg^{2+} and 8.0 mM ATP (initially); part B, 8.1 mM Mg^{2+} and 7.4 mM ATP (initially). The levels of added ADP are indicated next to each curve. The progress curves are displaced in time for clarity of presentation. For other details see the text: Discrimination between ADP and MgADP as inhibitors of nitrogenase.

present to chelate all the nucleotide in the assay (e.g. with 2.5 mM ATP plus 5 mM Mg). No creatine kinase or creatine-*P* was present in this assay. When an excess of Mg^{2+} was present (i.e. at 2.5 mM ATP plus 5 mM Mg^{2+}) inhibition by the product MgADP was too rapid to allow reliable measurements of initial oxidation rates.

Inhibitory effects of phosphate and ATP

The second product of ATP hydrolysis by nitrogenase is inorganic phosphate, which in high concentrations may bind a significant fraction of the total Mg^{2+} and could compete with ATP at a substrate binding site (see Discussion).

High levels of Mg^{2+} in coupled assays (up to 16 mM with 2.5 mM ATP) made the reaction somewhat more resitant to the inhibitory effects of added phosphate but phosphate was not found to be a more potent inhibitor than would be expected from the effect of it contribution to the ionic strength alone [13]. In assays using substrate level ATP such as shown in Fig. 4, high levels of Mg^{2+} (above 10—15 mM) resulted in precipitation of the enzyme unless equivalent amounts of ATP were present, so that we could not do experiments directly comparable to those of Thorneley and Willison [24]. Under the conditions of assay shown in Fig. 4, addition of inorganic phosphate at low levels (36 mM) stimulated the initial rate of dithionite oxidation and did not increase the degree of inhibition by accumulation of ADP, relative to an assay with no phosphate added.

We did not find that there was any significant inhibition by free ATP as de-

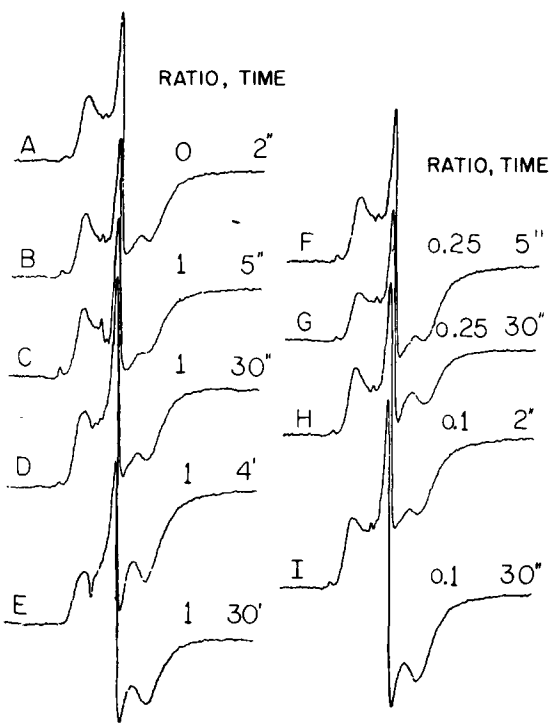

Fig. 5. Effect varying creatine kinase on the EPR signal of component II. Enzyme was the same mixture of components used for Fig. 2 experiments. Each EPR tube contained 9.0 μmol creatine phosphate, 0.75 μmol ATP, 1.50 μmol magnesium acetate, creatine kinase as indicated, sufficient nitrogenase to oxidize 625 nmol of dithionite per min (0.8 mg component I plus 1.7 mg component II) all in 300 μl containing 3 μmol potassium Morpholinopropane sulfonate (initial pH 7.4) and 6.25 μmol Tris · HCl (initial pH 8). EPR tubes containing everything but the nitrogenase component were flushed with N_2 and dithionite was added to give 15 mM final concentration. They were then incubated at 30°C until addition of the nitrogenase mixture. EPR spectra of the frozen samples were obtained under conditions as described by Orme-Johnson et al. [7]. All were obtained at 13°K using a power of 3 nW and the same relative gain. For each part of the figure we indicate the ratio of creatine kinase : nitrogenase determined as in Fig. 1 and Materials and Methods. The time from mixing to initiation of freezing is indicated for each part of the figure.

termined by a series of assays varying Mg^{2+} from 2 to 10 mM with constant ATP at 8 mM. There was, however, a more rapid onset of ADP inhibition with the high level of Mg^{2+} as shown in Fig. 4. A coupled assay system with 2.5 mM ATP and 5 mM Mg^{2+} gave a slightly greater initial velocity than these substrate level assays, even though the amount of ADP accumulated in the substrate level assays should be quite small over the short times concerned (~0.05mM after 1 min or 1/20 total ATP).

Effect of MgADP/MgATP on the EPR of nitrogenase

A number of EPR experiments were done to relate our observations of the activities of nitrogenase in standard assays to levels more closely approximating those found in intact nitrogen fixing organisms (Fig. 5), and to provide some information on the limitations of the ATP-generating system when EPR experiments are being done.

The general conditions of the system shown in Fig. 5 were the same as for Fig. 2 with varying levels of creatine kinase present in the assay, but levels of dithionite were higher and more nitrogenase was used. The detailed interpretation of the signal of component II at pH $\geqslant$ 8 in the ATP and ADP forms is discussed elsewhere [7]. After 2 s in the absence of creatine kinase (part A) component II was present as a mixture of ATP and ADP forms. With a high level of kinase present (165 μg/ml, part B) it was nearly fully in the ATP form at an even longer time. Compare the right trough in the two cases. After 30 s (part C) when it can be calculated from the ratio of nitrogenase/kinase that the enzyme was in steady state (see Fig. 2), the enzyme appeared to be primarily in the ADP form. After 4 min (part D) the signal of component I began to reappear. Thawing this same sample under vacuum and letting it stand at 30°C for 30 min under N_2 gave the spectrum shown in E. The notch on the low field side is part of the component I spectrum which has fully reappeared at this time [7]. Reducing the level of creatine kinase in the system resulted in a more rapid transition to the "steady-state" form of the enzyme which appeared to be primarily the ADP form. In parts F and G we show spectra, at 5 s and 30 s, respectively, for a lower level creatine kinase (40 μg/ml). In parts H and I we show 2 and 30 s, respectively, for one-tenth the initial amount of creatine kinase (16.5 μg/ml). Note that in each series the enzyme was largely present as the ADP form after 30 s (C, G, I) even though the component I signal did not reappear until $\gtrsim$ 4 min. At 30 s approximately one-third of the energy source was calculated to have been consumed.

Discussion

We have undertaken a reexamination of the role of the ATP-generating system in nitrogenase studies for three reasons. First, there have been differing choices of assay system between different laboratories and in some cases the assay systems themselves may lead to variation in results. Second, there have been differing interpretations of experiments performed with the same organism in very similar (but not identical) ways. Third, we have observed variations in activities using the same preparation of nitrogenase, upon changing the pH and buffer ions present in the assay system.

Kinetic properties of creatine kinase

With respect to the last point, we have made a systematic study of the variation in creatine kinase activity as a function of pH and buffer used (unpublished observations with P. Ludden). The differences obtained in V are relatively small (less than 50%) over the range of pH 6.6—7.5. Preparations of kinase from Brill, Tso, and Burris had activities ranging from 55 to 73 I.E.U./mg at pH 7.4 although the manufacturers data sheets indicated activities from 35 to 135 I.E.U./mg. Differing buffer systems were tested by the method of Morrison and Cleland [16] using simultaneous variation of both ADP and creatine-P. Although there was little effect of buffers or pH on V, there was a marked rise in K_m with increasing pH. This means that as pH increases, more enzyme will be required to keep the ADP level below any particular concentration which may be chosen as satisfactory for the study underway. For this reason, in many studies assumption that the generator is saturated is not correct. True specific activities of creatine kinase must be determined under the actual assay conditions being used.

Two other important considerations in the use of creatine kinase are its absolute requirement for the MgADP complex as substrate, and its sensitivity to dead-end inhibition by MgATP [20]. This means that the kinase will be ineffective if the Mg^{2+} concentration is insufficient to keep the ADP in a utilizable form, or if ATP levels are very high. From the results of Schimerlik and Cleland [20] at pH 7.0 and those of Morrison and James [21] at pH 8.0, one can interpolate the kinetic constants of creatine kinase at pH 7.4. Using the terminology and

TABLE I

FORMATION OF METAL-NUCLEOTIDE COMPLEXES IN THE NITROGENASE ASSAY

Fog MgATP and MgADP, values for stability constants were interpolated to an ionic strength of 0.160 at 30°C, others at ionic strength = 0.2. Results are calculated for a pH of 7.4 in a standard assay system with 2.5 mM ATP, 30 mM creatine-P initially. The pK for ionization of ATP at high ionic strength is 7.0 while that for ADP is 6.7 under these conditions [23]. The equilibrium constant $MgATP^{2+}$ + creatine$^{\pm}$ ⇌ $MgADP^{-}$ + creatine-$P^{\pm 2-}$ is taken as 10^{-3} for pH 7.4 [17,26].

Metal	Ligand	Stability constant	Concentrations (mM) with 5 mM Mg^{2+}		Concentrations (mM) with 2.5 mM Mg^{2+}	
			free nucleotide	liganded form	free nucleotide	liganded form
Mg^{2+}	ATP^{4-}	34 000 [a]	0.055	2.21	0.28	1.73
	ATP^{3-}	720 [a]	0.02	0.015	0.11	0.007
	ADP^{3-}	2 140 [a]	0.001	0.001	0.002	0.001
	ADP^{2-}	100 [a]	—	—	—	—
	Creatine-$P^{\pm}$	20 [a]	19.5	0.5	19.9	0.07
	P_i	76 [b]	9.2	0.82	9.9	0.14
$(Mg^{2+})_2$	ATP^{4-}	60 [c]	—	0.16	—	0.02
Mg^{2+}	$(ATP^{4-})_2$	300 [d]	—	0.04	—	0.16
K^{+}	ADP^{3-}	7 [b]	—	0.001	—	0.002
Na^{+}	ADP^{3-}	7 [b]	—	0.001	—	0.002

[a] Ref. 23.
[b] Ref. 25.
[c] Ref. 27.
[d] Ref. 26.

results of Schimerlik and Cleland [20] for binding constants, the K_a (Michaelis constant) for MgADP is 40 μM while the K_{ia} (dissociation constant) is 140 μM. Morrison and James [21] report a K_i of 0.25 mM for ADP^{3-} vs. $MgADP^-$. Thus when the concentration of Mg^{2+} is lower than the total nucleotide concentration there will be a proportionately greater inhibition of the creatine kinase generator by the competitive inhibitor ADP^{3-}, even when a "saturating" amount of creatine-*P* is present.

The dead-end inhibition constant (K_{Iq}) for E-*P*-creatine-MgATP is reported to be 3.2 mM [20]. Hence, there is a partial inhibition of the creatine kinase generator under our usual conditions for assay of nitrogenase where there is 2.5 mM ATP and 5 mM Mg^{2+}. The inhibition would be appreciably greater under the assay conditions of Eady et al. [10] or Bergerson and Turner [12].

Distribution of magnesium and free phosphorylated compounds

It would be tedious to derive the concentrations of all the nucleotide species (and potential inhibitors of nitrogenase and creatine kinase) for the wide variety of assay conditions in common use. It may, however, be instructive to provide an example for some typical assay conditions (Table I). Kuby and Noltmann [26] give a comprehensive discussion of such an approach to the creatine kinase system. Although their calculations were carried out prior to the recent studies on metal-nucleotide complexation [23] or studies on the mechanism of creatine kinase [16,20,22,28] their approach is valid even if some of the resulting numbers are not precisely correct.

If one tc consider all possible complexes a goodly amount of computing time is needed. Since, as is discussed below, some of the essential data is lacking for nitrogenase in vivo, e.g. the levelsof Mg^{2+}, distribution of nucleotide pools, source(s) of ATP, we have not attempted to develop a full program to deal with this enzyme. Rather we have examined the critical variables which are of practical interest in vitro and then asked the question, whether these appear to be important for the in vivo situation as presently understood. From the studies of Mg-nucleotide complex formation by Phillips et al. [23], one can similarly determine for a wide range of pH and ionic strength the relative distribution of adenosine phosphates and their magnesium chelates. Morrison and Heyde [28] have derived such tables at zero ionic strength for Mg^{2+} and pH values of common interest.

In the table we show sample calculations for one-third consumption of creatine-*P* with two different starting levels of Mg^{2+}. Note that the level of MgATP is appreciably lower when equimolar Mg^{2+} and ATP are used than when a 2-fold excess of Mg^{2+} over ATP is used. When equimolar Mg^{2+} and ATP are used in a potassium or sodium buffer of 100 mM, the potassium or sodium salt of ADP will be present at about three times the concentration of MgADP. It is important to note that these equilibrium calculations for ADP levels are not in very good agreement with the actual measured steady-state concentrations of total ADP and ATP in nitrogenase assay (c.f. Fig. 2). This is understandable because an assay can never be at equilibrium, since the action of nitrogenase effects a significant perturbation of the creatine kinase equilibrium.

Energy charge and nitrogenase

A question of central interest to us is what are the in vivo effectors of nitro-

genase activity. The use of an ATP-regenerating system, which we have shown to be essential for most practical studies of more than milliseconds duration, sets some severe constraints on the range over which we can vary nucleotide and Mg^{2+} concentrations, since the performance of the regenerating system becomes limiting outside a fairly narrow range, within which it acts as a "buffer".

Purich and Fromm [29] used the data of Morrison and James [21] to simulate the response of creatine kinase to energy charge. The concluded that "energy charge" in the particular sense in which Atkinson [30] has defined it ([ATP] [1/2ADP]/[AMP] + [ADP] − [ATP]) is probably not a critical variable in control of kinase activities although the ratio of MgADP/MgATP may be a controlling factor. Beginning with a total nucleotide level of 9.2 mM and creatine plus creatine-*P* of about 11 mM, they varied the ratio of creatine/creatine-*P* and the energy charge. The particular point of interest in their results (Figs. 6A and 6B in ref. 29) is that the net rate of ATP synthesis, in response to a perturbation of the equilibrium, drops off very sharply as the energy charge approaches 1.0, even when there is no creatine present and although the equilibrium of the reaction lies far toward ATP synthesis [17]. The diminished rate of ATP synthesis is primarily due to formation of abortive complexes, as discussed above, and the degree of inhibition depends critically on the concentration of Mg^{2+} and nucleotides as well as on the reaction mechanism assumed to be operating. For example, in the presence of 7.7 mM creatine and 4 mM creatine-*P* the activity of the kinase in the synthesis of ATP is only 1/20 *V* when the energy charge is 0.95 (which corresponds to a ratio of ADP/ATP about 1/10). This allows a proportional control of the kinase activity as a function of the ratio ADP/ATP in an approximately linear manner between energy charge of about 0.80 and 0.98. In Fig. 2 the maximum ratio of units of creatine kinase to the units of nitrogenase (expressed as the ATP hydrolysis rate) corresponds to about 20 : 1. Thus, from the response of the kinase to energy charge, the rate of ATP synthesis should equal the rate of ATP hydrolysis when the ratio of nucleotides is approximately 1/10 as observed in the assay (Fig. 2). Changing the level of kinase either upward or downward by 2-fold would produce only a small change in the steady-state level of ADP since as the ADP level decreased the activity of the kinase would decrease correspondingly, and as the level of ADP increased the kinase would increase its activity. Only when the ratio of creatine kinase : nitrogenase approaches 1 : 1 will we observe appreciable inhibition of nitrogenase activity, in "initial velocity" studies. The primary consequence of lowering the ratio creatine kinase : nitrogenase is to shorten the period of "linear" functioning of the nitrogenase. It should also be apparent from this discussion that truly linear nitrogenase activity will only be observed so long as the creatine kinase can maintain MgADP/MgATP below the ratio which affects nitrogenase activity. Because of the relative kinetic parameters of creatine kinase and nitrogenase the requirement is not fully satisfied in any assay. It is satisfactorily approximated under the conditions which we use for typical enzymatic assays (refs. 13, 22 and this work). It would also be satisfactory to use true initial velocities without any regenerating system present but this limits the time of observation to the millisecond region when very active nitrogenase is used as in EPR studies.

Practical consequences for assays

Several conclusions follow from our results, and the above analysis. First, Mg_2ATP inhibition is not a problem with *Clostridium pasteurianum* nitrogenase at reasonable levels of ATP and Mg^{2+} [24]. Free ATP does not appear to be an inhibitor of any significant potency when physiological levels of MgATP are present [27]. If ATP_2Mg is formed it must not be a strong inhibitor under these conditions either. Inorganic phosphate is not a specific inhibitor of *C. pasteurianum* nitrogenase. Indeed, Hermann [31] examined the phosphate inhibition of nitrogenase from *Bacillus polymyxa* by dithionite oxidation assay and found it to be uncompetitive with ATP. This inhibition pattern would suggest that it is competitive with creatine-*P* in the ATP-regenerating system. This would explain both the uncompetitive nature of the inhibition and our finding that phosphate stimulates dithionite oxidation in the presence of substrate levels of ATP. Our results emphasize the importance of understanding the limitations of the ATP-regenerating system in any attempt to interpret nitrogenase activity as a function of Mg^{2+} and nucleotide concentrations and other variables. When attempting to determine the apparent K_m of nitrogenase at very low levels of ATP, the relative K_{ia} of creatine kinase for ADP (140 μM [20]) vs. that of nitrogenase (5 μM [3]) may result in an inhibitory ratio of ADP/ATP even with a relatively large "calculated" excess of creatine kinase [8]. Similarly, use of excess ATP [9] or equimolar starting Mg^{2+} and ATP concentrations [12] leads to a rise in the total ADP since only the monomagnesium complex is a substrate for creatine kinase [22]. The free ADP so produced may inhibit nitrogenase to some extent.

At high pH the assay becomes non-linear when a smaller fraction of the creatine-*P* has been consumed for a comparable assay done at neutral pH [10]. Use of a high pH combined with high ionic strength in EPR experiments [14] resulted in a very long time being required for exhaustion of what was calculated to be a limiting amount of dithionite. It also leads, as we have shown, to the enzyme operating in a severely ADP-inhibited state at rather short times. This may in part account for the difference observed in the degree of reduction of the g 3.7 signal in steady-state EPR experiments of *Klebsiella pneumoniae* [14] as compared to *C. pasteurianum* or *Azotobacter vinelandii* [7] when activity of the enzyme was rate-limited by the activity of the Fe- protein component.

Substrate level ATP is really not a good alternative to the use of creatine kinase. When there is sufficient Mg^{2+} that most of the ATP is in the form MgATP (which is the true substrate), the inhibition by MgADP sets in very rapidly [9] and linear enzyme activity with time is not observed. Moustafa and Mortenson [1] and Kennedy et al. [5] obtained qualitatively correct results, that nitrogenase is inhitibed by ADP, but their experiments could not quantitatively show whether and to what extent the magnesium chelates of ADP and ATP were involved in the control of the activity. Ljones [4] was able to deduce something of the nature of the sensitivity of nitrogenase to MgADP/MgATP ratios by following progress curves for dithiomite oxidation. The rapid onset of inhibition by MgADP prevented reliable quantitation of the effect, however.

Apparent K_m for ATP

The apparent K_m for ATP of nitrogenase from different species has been reported in the range of 0.1—1 mM using a wide variety of assay techniques [1,4, 5,8—10]. The true dissociation constant K_{ia} has only been reported for *C. pasteurianum* [3] and is much lower than the K_m (20 μM vs. 500 μM for K_{ia} and K_m, respectively). If this proves to be a general phenomenon, association of nucleotide with the enzyme is not a rate-limiting step for catalysis [32]. This conclusion is supported by EPR experiments in which reduction of the enzyme into a steady-state occurs more rapidly then substrate reduction [7,33]. However, the ratio of substrate MgATP to inhibitor MgADP may determine the concentration of effective "super-reductant" Fe protein available to promote the next step(s) in the overall reaction, if the total concentration of Fe protein is not in large excess of that needed for its "effector function" [12]. A simple prediction from this is that the overall reaction should be more sensitive to ADP when the Fe protein is limiting the overall reaction. Thus our results in Fig. 3 might have shown two different lines for inhibitory effect of ADP/ADT ratio. That they did not show two apparently different slopes can only mean that both ratios of components were in a range where the observed activity was responsive to concentration of the Fe protein component. Appleby et al. [34] have found a linear relationship between nitrogenase activity and ADP/ATP ratio in intact Rhizobium bacteroids. The meaning of such a simple linear relationship as observed by them and ourselves is not apparent, since a competition between ATP and ADP should be logarithmic, if they are simple competitors.

There are species-dependent differences in nitrogenase response to component ratios. With *A. vinelandii* the observed specific activity of the Mo-Fe protein continues to increase until a very large excess of the Fe protein is present, whereas with *C. pasteurianum* there is a relatively wide range of component ratios over which the specific activity of the Mo-Fe protein is a constant (our unpublished observations). The component ratio dependence of different reactions (e.g. acetylene vs. N_2 reduction) is also markedly different [35] for *A. vinelandii*, as is the ATP dependence of the different reactions [8,35], so that a measured K_m is difficult to interpret in a simple way.

Implication for EPR studies of the creatine kinase : nitrogenase couple

Our original interest in the kinetics of creatine kinase behavior came out of our attempts to scale up nitrogenase assays for use in EPR experiments other than simple "steady-state" observations [7]. The results shown in Fig. 5 indicate the critical importance of creatine kinase in interpretation of the behavior of nitrogenase. Even if one uses rapid freeze quenching it is relatively easy to be observing the ADP form of the Fe protein, rather than the ATP form unless there is a good excess of creatine kinase over nitrogenase. An additional complication is that the EPR of the ATP form of the Fe protein does not differ from that of the uncomplexed form at pH 7 (our unpublished observations), even though it is known from the studies of Tso and Burris [3] that there is tight binding of ATP to the protein at this pH.

The problem posed by our results is that one really ought to repeat the original EPR experiments [6,7,14] at a pH nearer the optimum of the nitrogenase yet one cannot follow the response of the Fe protein to ATP vs. ADP at a pH

near the optimum for nitrogenase activity, at least in *C. pasteurianum*. It should also be remembered that the pH of the operation may not be the pH for observation since most of the buffers commonly used have a large temperature coefficient. For instance Tris · HCl, from an assay run at 30°C with a nominal pH of 7.1 would have an effective pH of 8.0 if samples were frozen slowly in liquid nitrogen, giving rise to the ATP form of the Fe protein which is completely absent from a sample of the protein frozen quickly by plunging in cold isopentane. This effect complicates the analysis of experiments such as those of Zumft et al. [36] who used tris(hydroxymethyl)methylaminomethane buffer (temperature coefficient −0.02 pH/degree [32]) at pH 7.5 and froze them slowly. The Fe protein clearly undergoes a conformation change on binding ATP at high pH [7]. Whether this is pertinent to the enzyme mechanism remains to be demonstrated.

Nitrogenase in vivo

Haaker et al. [2] have recently measured both pyridine nucleotide and ADP/ATP levels in *Azotobactor* under O_2-limited metabolic regimes. Reduction of acetylene, used as a measure of dinitrogen-fixing capability, depends critically on the ADP/ATP ratio, and relatively little on the concentration of reduced pyridine nucleotides. Use of respiratory inhibitors and acridine dyes to measure energization of the cytoplasmic membrane indicated that the source of electrons is the energized membrane. Under normal growth conditions it is the ADP/ATP ratio which will control nitrogenase, and indeed the critical point for turn-on of nitrogenase appeared to be at a ratio of adenine nucleotides near 1 : 1, as found in our in vitro studies, (see Fig. 3) and by others [1,5,34]. Appleby et al. [34] have used similar techniques to control respiration in intact bacteroids of soybean and reached similar conclusions.

One considerable difficulty, yet to be resolved, is that of measuring levels of magnesium in whole cells and determining the segregation of magnesium into metabolic pools. The good agreement between our results, and those of Haaker et al. [2] and Appleby et al. [34] strongly suggest that in these organisms there is sufficient magnesium present in the relevant adenine nucleotide pool so that essentially all of the ADP is in an effectively inhibitory form. This is reasonable since the obligatory substrate of nitrogenase is MgATP and there must be equimolar amounts of magnesium and ADP as products of the hydrolysis. There should thus be primarily MgADP near the active site of the nitrogenase and the rate of regeneration of ATP would be the limiting step for the nitrogenase activity.

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